10/073,293

РОССИЙСКОЕ АГЕНТСТВО ПО ПАТЕНТАМ И ТОВАРНЫМ ЗНАКАМ
(РОСПАТЕНТ)
ФЕДЕРАЛЬНЫЙ ИНСТИТУТ ПРОМЫШЛЕННОЙ СОБСТВЕННОСТИ

рег.No 20/12- 90 "11"февраля 2002 г.

С П Р А В К А

Федеральный институт промышленной собственности Российского агентства по патентам и товарным знакам настоящим удостоверяет, что приложенные материалы являются точным воспроизведением первоначального описания, формулы и чертежей (если имеются) заявки на выдачу патента на изобретение № 2001104998, поданной в феврале месяце 26 дня 2001 года (26.02.2001).

Название изобретения	METHOD FOR PRODUCING L-AMINO ACID USING BACTERIA BELONGING TO THE GENUS ESCHERICHIA
Заявитель	Закрытое акционерное общество «Научно-исследовательский институт Аджиномото-Генетика»
Действительный автор(ы)	ТАБОЛИНА Екатерина Александровна РЫБАК Константин Вячеславович ХУРГЕС Евгений Моисеевич

CERTIFIED COPY OF
PRIORITY DOCUMENT

Уполномоченный заверить копию
заявки на изобретение

А.Л. Журавлев
Заведующий отделом

METHOD FOR PRODUCING L-AMINO ACID USING BACTERIA BELONGING TO THE GENUS ESCHERICHIA

Technical field

The present invention relates to biotechnology, specifically to a method for producing L-amino acids by fermentation and more specifically to genes derived from bacteria *Escherichia coli*. The genes are useful for improvement of L-amino acid productivity, for example, L-arginine.

Background art

Conventionally the L-amino acids have been industrially produced by method of fermentation utilizing strains of microorganisms obtained from natural sources or mutant of the same especially modified to enhance L-amino acid productivity.

There have been disclosed many techniques to enhance L-amino acid productivity, for example, by transformation of microorganism by recombinant DNA (see, for example, US patent No. 4,278,765). These techniques based on the increasing of activities of the enzymes involved into amino acid biosynthesis and/or desensitizing the target enzymes to the feedback inhibition by produced L-amino acid (see, for example, Japanese Laid-open application No56-18596 (1981), WO 95/16042 or US patent Nos. 5,661,012 and 6,040,160).

On the other hand, increased L-amino acid excretion can enhance the productivity of strain producing L-amino acid. Strain of bacterium belonging to the genus *Corynebacterium* having increased expression of L-lysine excretion gene (*lysE* gene) is disclosed (WO 9723597A2). In addition, genes coding for the efflux proteins suitable for secretion of L-cysteine, L-cystine, N-acetylserine or thiazolidine derivatives are also disclosed (USA Patent No. 5,972,663).

At present several *Escherichia coli* genes coding for putative membrane proteins enhancing L-amino acid production are disclosed. Additional copy of *rhtB* gene makes a bacterium more resistant to L-homoserine and enhances production of L-homoserine, L-threonine, L-alanine, L-valine and L-isoleucine (European patent application EP994190A2). Additional copy of *rhtC* gene makes a bacterium more resistant to L-homoserine and L-threonine and enhances production of L-homoserine, L-threonine and L-leucine (European patent application EP1013765A1). Additional copy of *yahN*, *yeaS*, *yfiK* and *yggA* genes enhance production of L-glutamic acid, L-

lysine, L-threonine L-alanine, L-histidine, L-proline, L-arginine, L-valine and L-isoleucine (European patent application EP1016710A2). And though complete genome sequence of *Escherichia coli* strain K-12 is described (Blattner F.R., Plunkett G., Bloch C.A. et al., Science, 227, 1453-1474, 1997; ftp://ftp.genetics.wisc.edu/pub/sequence/ecolim52.seq.gz), there are many ORFs, the function of which still remain unknown.

Disclosure of the invention

An object of present invention is to enhance the productivity of L-amino acid producing strains and to provide a method for producing L-amino acid, for example, L-arginine, using the strains.

This aim was achieved by identifying genes coding for proteins, which are not involved into biosynthetic pathway of target L-amino acid but enhance its production. An example of such protein could be a membrane protein having L-amino acid excretion activity. Based on the analysis of complete genome sequence of *Escherichia coli,* proteins with 4 or more putative transmembrane segments (TMS) were selected. As a result of diligent screening, the present inventors have identified one gene among them, that is b3434, and thoroughly studied it. The gene b3434 has been known as putative CDS which may encode functionally unknown protein (numbers 1463 to 2056 in the sequence of GenBank accession AE000420 U00096). The gene b3434 is also known as *yhgN*. Also the present inventors have found that by enhancing the activity of the protein encoded by b3434 gene the productivity of L-amino acid producing strain is enhanced. Thus the present invention has been completed.

The present inventions are as follows:

1). An L-amino acid producing bacterium belonging to the genus *Escherichia,* wherein L-amino acid production by the bacterium is enhanced by enhancing an activities of proteins as defined in the following (A) or (B) in a cell of the bacterium:

(A) a protein which comprises the amino acid sequence shown in SEQ ID NO:3 in Sequence listing;

(B) a protein which comprises an amino acid sequence including deletion, substitution, insertion or addition of one or several amino acids in the amino acid sequence shown in SEQ ID NO:3 in Sequence listing, and which has an activity of making bacterium having enhanced resistance to the L-amino acids and/or its analogs, such as DL-o-methylserine and 6-

diazo-5-oxo-L-norleucine, and having enhanced sensitivity to S-(2-aminoethyl)cysteine;

(hereinafter, the proteins as defined in the above (A) or (B) are referred to as "proteins of the present invention")

2). The bacterium according to the above bacterium, wherein the activities of the proteins as defined in (A) or (B) are enhanced by transformation of the bacterium with a DNA coding for the proteins as defined in (A) or (B), or by alteration of expression regulation sequence of said DNA on the chromosome of the bacterium.

3). The bacterium according to the above bacterium, wherein the transformation is performed with a multicopy vector.

4). A method for producing L-amino acid, which comprises cultivating the bacterium according to the above bacterium in a culture medium and collecting from the culture medium L-amino acid to be produced and accumulated.

5). The method according to the above method, wherein L-amino acid is L-arginine.

6). The method according to the above method, wherein the bacterium has enhanced expression of arginine regulon.

The method for producing L-amino acid includes production of L-arginine using L-arginine producing bacterium wherein activities of the proteins of the present invention such as that comprising amino acid sequence shown in SEQ ID NO:3 are enhanced.

The present invention will be explained in detail below.

The bacterium of the present invention is an L-amino acid producing bacterium belonging to the genus *Escherichia,* wherein L-amino acid production by the bacterium is enhanced by enhancing an activity of the proteins of the present invention in a cell of the bacterium.

A bacterium of present invention is L-amino acid producing bacterium belonging to the genus *Escherichia* having enhanced activities of proteins, which enhance the productivity of the target L-amino acid. Concretely the bacterium of present invention is L-amino acid producing bacterium belonging to the genus *Escherichia* which has enhanced activities of the proteins of the present invention. More concretely the bacterium of present invention harbors the DNA having

overexpressed b3434 gene on chromosomal DNA or plasmid in the bacterium and has enhanced ability to produce L-amino acid, for example, L-arginine.

The proteins of the present invention include ones as defined in the following (A) or (B):

(A) a protein which comprises the amino acid sequence shown in SEQ ID NO:3 in Sequence listing;

(B) a protein which comprises an amino acid sequence including deletion, substitution, insertion or addition of one or several amino acids in the amino acid sequence shown in SEQ ID NO:3 in Sequence listing, and which has an activity of making bacterium having enhanced resistance to the L-amino acids and/or its analogs;

The number of "several" amino acids differs depending on the position or the type of amino acid residues in the three-dimensional structure of the protein It may be 2 to 20, preferably 2 to 10, and more preferably 2 to 5 for the protein (A).

Resistance to L-amino acids and/or its analogs means ability for bacterium to grow on a minimal medium containing L-amino acid or its analog in concentration under which the wild type or the parental strain of the bacterium cannot grow, or ability for bacterium to grow faster on a medium containing L-amino acid or its analog than the wild type or the parental strain of the bacterium. L-amino acid analogs are exemplified by DL-o-methylserine, 6-diazo-5-oxo-L-norleucine, DL-β-hydroxy-norvaline or the like. Above mentioned concentration of L-amino acid or its analog is generally 1100 to 9600 μg/ml, preferably 3000 to 3500 in case of DL-o-methylserine, generally 5 to 50 μg/ml, preferably 12 to 18 in case of 6-diazo-5-oxo-L-norleucine and generally 25 to 250 μg/ml, preferably 70 to 90 μg/ml in case of DL-β-hydroxy-norvaline.

Sensitivity to L-amino acids and/or its analogs means ability for bacterium to grow in longer proliferation time than its parental strain or the wild type strain on a minimal medium containing a concentration of L-amino acid or its analog. Alternatively, sensitivity to L-amino acids and/or its analogs means ability for bacterium not to grow on a minimal medium containing L-amino acid or its analog in a concentration under which the wild type or the parental strain of the bacterium grow. Such L-amino acid analog is exemplified by S-(2-aminoethyl)cysteine. Above mentioned concentration is generally 0.2 to 2.0 μg/ml, preferably 0.5 to 1.0 μg/ml in case of S-(2-aminoethyl)cysteine.

The bacterium of the present invention also includes one wherein the activities of the proteins of the present invention are enhanced by transformation of said bacterium with DNA coding for protein as defined in (A) or (B), or by alteration of expression regulation sequence of said DNA on the chromosome of the bacterium.

The DNA, which is used for modification of the bacterium of the present invention, codes for putative membrane protein. Concretely the DNA codes for protein having 4 or more transmembrane segments. Such DNA may code for proteins having L-amino acid excretion activity. More concretely, the DNA is represented by b3434 gene. The b3434 gene can be obtained by, for example, PCR using primers having nucleotide sequence shown in SEQ ID No: 1 and 2.

Analysis of complete genome sequence of *Escherichia coli* allowed to select the genes coding for proteins having 4 or more putative TMS. Proteins with known function and transporters described by Paulsen I.T., Sliwinski M.I., Saier M.H. (*J.Mol.Biol.*, 1998, 277, 573) and Linton K.J., Higgins C.F.(*Molecular Microbiology*, 1998, 28(1), 5) were excluded from the group to be screened. As a result of diligent screening among the rest of genes, several genes coding for putative membrane exporters were chosen. And it was found the overexpression of b3434 gene enhances the L-amino acid production by L-amino acid producing strain.

The DNA of the present invention includes a DNA coding for the protein which include deletion, substitution, insertion or addition of one or several amino acids in one or more positions on the protein (A) as long as they do not lose the activity of the protein. Although the number of "several" amino acids differs depending on the position or the type of amino acid residues in the three-dimensional structure of the protein, it may be 2 to 20, preferably 2 to 10, and more preferably 2 to 5 for the protein (A). The DNA coding for substantially the same protein as the protein defined in (A) may be obtained by, for example, modification of nucleotide sequence coding for the protein defined in (A) using site-directed mutagenesis so that one or more amino acid residue will be deleted, substituted, inserted or added. Such modified DNA can be obtained by conventional methods using treatment with reagents and conditions generating mutations. Such treatment includes treatment the DNA coding for proteins of present invention with hydroxylamin or treatment the bacterium harboring the DNA with UV irradiation or reagent such as N-methyl-N'-nitro-N-nitrosoguanidine or nitrous acid.

The DNA of the present invention include variants which can be found in the different strains and variants of bacteria belonging to the genus *Escherichia* according to natural diversity. The DNA coding for such variants can be obtained by isolating the DNA, which hybridizes with b3434 gene or part of the gene under the stringent conditions, and which codes the protein enhancing L-amino acid production. The term "stringent conditions" referred to herein is a condition under which so-called specific hybrid is formed, and non-specific hybrid is not formed. For example, the stringent conditions includes a condition under which DNAs having high homology, for instance DNAs having homology no less than 70% to each other, are hybridized. Alternatively, the stringent conditions are exemplified by conditions which comprise ordinary condition of washing in Southern hybridization, e.g., 60°C, 1 x SSC, 0.1% SDS, preferably 0.1 x SSC, 0.1% SDS. As a probe for the DNA which codes for variants and hybridizes with b3434 gene, a partial sequence of the nucleotide sequence of SEQ ID NO: 3 can also be used. Such a probe may be prepared by PCR using oligonucleotides produced based on the nucleotide sequence of SEQ ID NO: 3 as primers, and a DNA fragment containing the nucleotide sequence of SEQ ID NO: 3 as a template. When a DNA fragment in a length of about 300 bp is used as the probe, the conditions of washing for the hybridization consist of, for example, 50°C, 2 x SSC, and 0.1% SDS.

Transformation of bacterium with DNA coding for protein means introduction of the DNA into bacterium cell for example by conventional methods to increase expression of the gene coding for the protein of present invention and to enhance the activity of the protein in the bacterial cell.

Techniques for enhancement of gene expression includes methods increasing the gene copy number. Introduction of a gene into a vector that is able to function in a bacterium belonging to the genus *Escherichia* increases copy number of the gene. For such purposes multi-copy vectors can be preferably used. The multi-copy vector is exemplified by pBR322, pMW119, pUC19, pET22b or the like.

Besides, enhancement of gene expression can be achieved by introduction of multiple copies of the gene into bacterial chromosome by, for example, method of homologous recombination or the like.

In case that expression of two or more genes is enhanced, the genes may be harbored together on the same plasmid or separately on different plasmids. It is also

acceptable that one of the genes is harbored on a chromosome, and the other gene is harbored on a plasmid.

On the other hand, enhancement of gene expression can be achieved by locating the DNA of the present invention under control of a potent promoter. For example, *lac* promoter, *trp* promoter, *trc* promoter, P_L promoter of lambda phage are known as potent promoters. Using the potent promoter can be combined with multiplication of gene copies.

The bacterium of the present invention can be obtained by introduction of the aforementioned DNAs into bacterium inherently having ability to produce L-amino acid. Alternatively, the bacterium of present invention can be obtained by imparting ability to produce L-amino acid to the bacterium already harboring the DNAs. For the parent strain which is to be enhanced in activity of the proteins of the present invention, L-arginine producing bacteria belonging to the genus *Escherichia* such as strains AJ11531 and AFJ11538 (JP56106598A2), AJ11593 (FERM P-5616) and AJ11594 (FERM P-5617) (Japanese Patent Laid-open No. 57-5693) or the like can be employed.

The bacterium of the present invention may be further enhanced expression of one or more genes which is involved in L-amino acid biosynthesis. Such gene is exemplified by arginine regulon, which preferably comprises a gene encoding N-acetylglutamate synthase of which feedback inhibition by L-arginine is desensitized (Rajagopal B.S. et al, Appl. Environ. Microbiol., 1998, v.64, No.5, p.1805-1811).

The method of present invention includes method for producing L-arginine, comprising steps of cultivating the bacterium of the present invention in a culture medium, to allow L-arginine to be produced and accumulated in the culture medium, and collecting L-arginine from the culture medium.

In the present invention, the cultivation, the collection and purification of L-amino acid from the medium and the like may be performed in a manner similar to the conventional fermentation method wherein an amino acid is produced using a microorganism. A medium used for culture may be either a synthetic medium or a natural medium, so long as the medium includes a carbon source and a nitrogen source and minerals and, if necessary, appropriate amounts of nutrients which the microorganism requires for growth. The carbon source may include various carbohydrates such as glucose and sucrose, and various organic acids. Depending on the mode of assimilation of the used microorganism, alcohol including ethanol and

glycerol may be used. As the nitrogen source, various ammonium salts such as ammonia and ammonium sulfate, other nitrogen compounds such as amines, a natural nitrogen source such as peptone, soybean-hydrolysate and digested fermentative microorganism are used. As minerals, potassium monophosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, calcium chloride, and the like are used.

The cultivation is performed preferably under aerobic conditions such as a shaking culture, and stirring culture with aeration, at a temperature of 20 to 40 °C, preferably 30 to 38 °C. The pH of the culture is usually between 5 and 9, preferably between 6.5 and 7.2. The pH of the culture can be adjusted with ammonia, calcium carbonate, various acids, various bases, and buffers. Usually, a 1 to 5-day cultivation leads to the accumulation of the target L-amino acid in the liquid medium.

After cultivation, solids such as cells can be removed from the liquid medium by centrifugation or membrane filtration, and then the target L-amino acid can be collected and purified by ion-exchange, concentration and crystallization methods.

Brief description of the drawing

Figure 1 shows the construction of plasmid pΔlacZ.

Best Mode for Carrying out the Invention

The present invention will be more concretely explained below with reference to Examples. In the Examples an amino acid is of L-configuration unless otherwise noted.

Example 1: Cloning of the b3434 gene on the plasmid pΔlacZ.

For cloning of the b3434 gene the vector pΔlacZ was used. Vector pΔlacZ is a derivative of the vector pET-22b(+) (Novagen, Madison, WI, USA). pET-22b(+) was treated by *Bgl*II and *Xba*I and ligated with polymerase chain reaction (PCR) fragment of plasmid pMB9-*lac* (Fuller F., *Gene*, 19, 43-54, 1982) treated with the same restrictases and carried P_{lac} UV5 promoter. For amplifying the P_{lac} UV5 promoter fragment by PCR primers having sequence depicted in SEQ ID Nos: 5 and 6 were used. The resulted plasmid was supplemented with structural part of *lacZ* gene (237 bp without promoter) by cloning *Sal*I-*Bam*HI fragment of the plasmid pJEL250

(Dymakova E. *et al.*, *Genetika* (rus), *35*, 2, 181-186, 1999). Scheme for obtaining vector pΔlacZ is shown in Figure 1.

The initial material for cloning of *E.coli* b3434 putative reading frame (b3434 gene) was the PCR fragment, which was obtained using DNA from *E. coli* strain TG1 as a template. For synthesis of this fragment two primers having sequence depicted in SEQ ID Nos: 1 and 2 were used. PCR was carried out on "Perkin Elmer GeneAmp PCR System 2400" under the following conditions: 40 sec. at 95 °C, 40 sec. at 47 °C, 40 sec. at 72 °C, 30 cycles. Thus, the 647 bp linear DNA fragment contained b3434 gene was obtained. This PCR fragment was treated by *Xba*I and *Bam*HI restrictases and inserted into multicopy vector pΔlacZ previously treated by the same restrictases.

Resulted plasmid with the PCR fragment was named pYHGN and carried b3434 gene under the control of the lactose promoter (P_{lac} UV5).

Example 2: The influence of the amplified b3434 gene on resistance of *E. coli* strain TG1 to amino acids and its analogs.

E. coli strain TG1(pYHGN) and TG1(pΔlacZ) strain having a vector without insertion (control strain) were grown overnight on LB medium supplemented with ampicilline (100 μg/ml). The night cultures of all strains were diluted at 25 times in fresh LB medium supplemented with ampicilline (100 μg/ml) and IPTG (0.5 mM) and were incubated 2 hours at 37 °C with aeration. The log phase cultures were diluted in 0,9% solution of NaCl and about 1000 cells were seeded on plates with solid Adams medium supplemented with ampicilline (100 μg/ml), IPTG (0.5 mM) and amino acid or its analog. After 2 – 4 days incubation at 37 °C the differences in colony size or colony number between the TG1 strain with hybrid plasmid and control TG1(pΔlacZ) strain were registered. The results of experiments are presented in Table 1.

Table 1.

Inhibitors	Concentration in media, μg/ml	Effect on the growth of TG1 strain having plasmid pYHGN
Proline	30000	no
3,4-Dihydroproline	23	no
Isoleucine	18000	no
DL-Thiaisoleucine	1	no
o-Methylthreonine	6	no
L-Serine	2800	no

DL-Serine	3600	no
DL-Serine hydroxamate	140	no
DL-o-Methylserine	3200	R
4-Azaleucine	45	no
6-Diazo-5-oxo-L-norleucine	15	R
Valine	7	no
Methionine	38000	no
Norleucine	500	no
Cysteine	1600	no
Homoserine	1000	no
DL-β-Hydroxy-norvaline	80	R
L-Aspartic acid β-hydroxamate	100	no
Arginine	4300	no
Lysine	5000	no
S-(2-Aminoethyl)cysteine	0.75	S
Histidine	3000	no
L-Histidine hydroxamate	200	no
DL-1,2,4-Triazole-3-alanine	80	no
Phenylalanine	13000	no
p-Fluorophenylalanine	6	no
L-o-Fluorophenylalanine	1.7	no
DL-o-Fluorophenylalanine	6	no
Tryptophan	12500	no
DL-4-Fluorotryptophan	0.1	no
4-Methyltryptophan	0.25	no
7-Methyltryptophan	100	no
DL-a-Methyltrypptophan	400	no
m-Fluoro-DL-tyrosine	0.5	no

no - no differences compare to the control strain
R - more colonies or colony size compare to the control strain
S – less colonies or colony size compare to the control strain

Example 3: Production of arginine by a strain having plasmids pYHGN.

The arginine producing strain 382 was transformed by the plasmid pYHGN carried the b3434 gene under the control of P_{lac} UV5 promoter. The strain 382 has been deposited in the Russian National Collection of Industrial Microorganisms (VKPM) (Russia 113545 Moscow 1 Dorozhny proezd, 1) on April 10, 2000 under accession number VKPM B - 7926.

The 5 colonies of each strain 382, 382(pΔlacZ) as a control strain contained plasmid without insertion and 382(pYHGN) were suspended in 2 ml of minimal medium ($(NH_4)_2SO_4$ – 25.0 g/l, K_2HPO_4 - 2.0 g/l, $MgSO_4$ $7H_2O$- 1.0 g/l, thiamin - 0.2 mg/l, yeast extract – 5 g/l, glucose - 60 g/l, ampicilline - 100 mg/l, if necessary) in 20-ml test tubes and were incubated overnight with aeration at 32 °C. The 0.2 ml of each night culture was transferred to the three 20-ml test tubes with 2 ml of fresh medium

for fermentation with or without IPTG and cultivated at 32 °C for 72 hours with rotary shaker.

Fermentation medium composition:

$(NH_4)_2SO_4$	25 g/l,
K_2HPO_4	2.0 g/l,
$MgSO_4\ 7H_2O$	1.0 g/l,
Thiamin	0.2 mg/l,
Yeast extract	5 g/l
Glucose	60 g/l,
$CaCO_3$	20 g/l
Ampicilline	100 mg/l, if necessary
IPTG	0.5 mM, if necessary

After cultivation the plasmid stability and optical absorbance of the medium at 540 nm were determined by conventional methods. Accumulated amount of arginine in the medium was determined by TLC. Liquid phase composition for TLC was as follows: isopropanol - 80 ml, ethylacetate - 40 ml, NH_4OH (30 %) - 25 ml, H_2O - 50 ml. The results are shown in Table 2. As it is seen, the hybrid plasmid pYHGN improved the arginine accumulation by the arginine producing strain 382.

Table 2

E. coli 382 with plasmid	IPTG	OD_{540}	Arg, g/l	Arg/OD
No	-	20	8.5	0.43
	+	22	6.7	0.31
pΔlacZ	-	28	6.3	0.23
	+	26	5.4	0.21
pYHGN	-	24	5.8	0.24
	+	26	9.3	0.36

SEQUENCE LISTING

<110>

<120> Method For Producing L-Amino Acid Using Bacterium Belonging to The Genus Escherichia

<130>

<140>
<141> 2001- -

<160> 8

<170> PatentIn Ver. 2.0

<210> 1
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 1
ggtctagagt ccgcggcaat tatcaggg 28

<210> 2
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 2
ccagatctgg tagttgtgac gctaccggg 29

<210> 3
<211> 594
<212> DNA
<213> Escherichia coli

<220>
<221> CDS
<222> (1)..(591)

<400> 3

```
atg aat gaa atc att tct gca gca gtt tta ttg atc ctg att atg gat   48
Met Asn Glu Ile Ile Ser Ala Ala Val Leu Leu Ile Leu Ile Met Asp
  1               5                  10                  15
ccg ctc gga aac cta cct att ttc atg tcc gta ctg aaa cat act gaa   96
Pro Leu Gly Asn Leu Pro Ile Phe Met Ser Val Leu Lys His Thr Glu
             20                  25                  30
ccg aaa aga cgg cgg gca atc atg gtg cga gag ttg ctt att gct ctc   144
Pro Lys Arg Arg Arg Ala Ile Met Val Arg Glu Leu Leu Ile Ala Leu
         35                  40                  45
ctg gtg atg ctg gtg ttc ctg ttt gcg ggt gag aaa att ctg gca ttt   192
Leu Val Met Leu Val Phe Leu Phe Ala Gly Glu Lys Ile Leu Ala Phe
```

```
     50                  55                  60
ctt agc cta cga gca gaa acc gtc tcc att tct ggc ggc atc att ctg   240
Leu Ser Leu Arg Ala Glu Thr Val Ser Ile Ser Gly Gly Ile Ile Leu
 65                  70                  75                  80
ttt ctg atc gcc att aaa atg att ttc ccc agc gct tca gga aat agc   288
Phe Leu Ile Ala Ile Lys Met Ile Phe Pro Ser Ala Ser Gly Asn Ser
                85                  90                  95
agc ggg ctt ccg gca ggt gaa gag cca ttt atc gtg ccg ttg gca att   336
Ser Gly Leu Pro Ala Gly Glu Glu Pro Phe Ile Val Pro Leu Ala Ile
            100                 105                 110
ccg tta gtc gcc ggg ccg act att ctc gcc acg ctg atg ttg ttg tct   384
Pro Leu Val Ala Gly Pro Thr Ile Leu Ala Thr Leu Met Leu Leu Ser
        115                 120                 125
cat cag tac ccg aat cag atg ggg cat ctg gtg att gct ctg ctg ctg   432
His Gln Tyr Pro Asn Gln Met Gly His Leu Val Ile Ala Leu Leu Leu
    130                 135                 140
gcc tgg ggc ggc acc ttt gtc atc ctg cta cag tct tcg cta ttt tta   480
Ala Trp Gly Gly Thr Phe Val Ile Leu Leu Gln Ser Ser Leu Phe Leu
145                 150                 155                 160
cgt ctg ctg ggc gag aaa ggg gtg aac gca ctt gaa cgc ctg atg gga   528
Arg Leu Leu Gly Glu Lys Gly Val Asn Ala Leu Glu Arg Leu Met Gly
                165                 170                 175
ttg att ctg gtg atg atg gca acc cag atg ttc ctc gac ggc att cga   576
Leu Ile Leu Val Met Met Ala Thr Gln Met Phe Leu Asp Gly Ile Arg
            180                 185                 190
atg tgg atg aag ggg taa                                          594
Met Trp Met Lys Gly
        195
```

<210> 4
<211> 197
<212> PRT
<213> Escherichia coli

<400> 4

```
Met Asn Glu Ile Ile Ser Ala Ala Val Leu Leu Ile Leu Ile Met Asp
  1               5                  10                  15
Pro Leu Gly Asn Leu Pro Ile Phe Met Ser Val Leu Lys His Thr Glu
            20                  25                  30
Pro Lys Arg Arg Arg Ala Ile Met Val Arg Glu Leu Leu Ile Ala Leu
        35                  40                  45
Leu Val Met Leu Val Phe Leu Phe Ala Gly Glu Lys Ile Leu Ala Phe
    50                  55                  60
Leu Ser Leu Arg Ala Glu Thr Val Ser Ile Ser Gly Gly Ile Ile Leu
65                  70                  75                  80
Phe Leu Ile Ala Ile Lys Met Ile Phe Pro Ser Ala Ser Gly Asn Ser
                85                  90                  95
Ser Gly Leu Pro Ala Gly Glu Glu Pro Phe Ile Val Pro Leu Ala Ile
            100                 105                 110
Pro Leu Val Ala Gly Pro Thr Ile Leu Ala Thr Leu Met Leu Leu Ser
        115                 120                 125
His Gln Tyr Pro Asn Gln Met Gly His Leu Val Ile Ala Leu Leu Leu
    130                 135                 140
Ala Trp Gly Gly Thr Phe Val Ile Leu Leu Gln Ser Ser Leu Phe Leu
145                 150                 155                 160
Arg Leu Leu Gly Glu Lys Gly Val Asn Ala Leu Glu Arg Leu Met Gly
                165                 170                 175
Leu Ile Leu Val Met Met Ala Thr Gln Met Phe Leu Asp Gly Ile Arg
            180                 185                 190
Met Trp Met Lys Gly
        195
```

<210> 5
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 5
cctttggtac cagatctgcg ggcagtgagc gcaacgc 37

<210> 6
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 6
ctgtttctag atcctgtgtg aaattgttat ccgc 34

What is claimed is:

1. An L-amino acid producing bacterium belonging to the genus *Escherichia* wherein L-amino acid production by said bacterium is enhanced by enhancing activities of proteins as defined in the following (A) or (B) in a cell of said bacterium:

(A) a protein which comprises the amino acid sequence shown in SEQ ID NO:3 in Sequence listing;

(B) a protein which comprises an amino acid sequence including deletion, substitution, insertion or addition of one or several amino acids in the amino acid sequence shown in SEQ ID NO:3 in Sequence listing, and which has an activity of making bacterium having enhanced resistance to L-amino acids and/or its analogs, such as DL-o-methylserine and 6-diazo-5-oxo-L-norleucine, and having enhanced sensitivity to S-(2-aminoethyl)cysteine;

2. The bacterium according to the claim 1, wherein said activities of proteins as defined as (A) or (B) are enhanced by transformation of said bacterium with DNA coding for protein as defined in (A) or (B), or by alteration of expression regulation sequence of said DNA on the chromosome of the bacterium.

3. The bacterium according to the claim 2, wherein the transformation is performed with a multicopy vector.

4. A method for producing L-amino acid, which comprises cultivating the bacterium according to any of claims 1 to 3 in a culture medium and collecting from the culture medium L-amino acid to be produced and accumulated.

5. The method according to claim 4, wherein L-amino acid is L-arginine.

6. The method according to claims 5, wherein the bacterium has enhanced expression of arginine regulon.

Abstract of disclosure

There is provided a method for producing L-arginine using bacterium belonging to the genus *Escherichia* wherein L-amino acid productivity of said bacterium is enhanced by enhancing an activity of proteins coded by b3434 gene.

Figure 1. Scheme for construction plasmid pΔlacZ.